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ANTIHISTOMONAL EFFECT OF ARTEMISININ AND *ARTEMISIA ANNUA* EXTRACTS IN EXPERIMENTALLY INFECTED TURKEYS.

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Summary

In the present investigation, previously established clonal cultures of *Histomonas meleagridis* and an infection model for histomonosis were used to evaluate the effect of five different *Artemisia annua* derived materials (i.e. dry leaves, artemisinin; and hexane, dichloromethane or methanol extracts). Successful *in vitro* inhibition of *H. meleagridis* clones were observed in all tested materials, except for the methanol extract. Whereas no activity against xenic bacteria accompanying the six *H. meleagridis* clones was observed. The dichloromethane extract of dry leaves (Ext-DCM) and artemisinin displayed the most promising antihistomonal properties and both were subsequently evaluated in a standardized experimental infection model in turkeys. Supplementation of Ext-DCM or artemisinin were commenced from day one of life and the birds were subsequently infected with clonal *H. meleagridis* at 2 weeks of age. Regardless of treatment, all infected turkeys showed severe clinical histomonosis and demonstrated severe typhlohepatitis.

Introduction

Infection with *Histomonas meleagridis* in poultry flocks has re-emerged since the ban of drugs providing effective prophylaxis and treatments (McDougald, 2005). Within recent years, efforts have been made to find alternatives to chemotherapeutics to combat histomonosis. Despite this, only a limited number

of *in vitro* studies on the effects of natural compounds on *H. meleagridis* are available (Zenner *et al.*, 2003; Grabensteiner *et al.*, 2007; Hauck & Hafez, 2007; Arshad *et al.*, 2008; Grabensteiner *et al.*, 2008; van der Heijden & Landman, 2008a). The situation is similar when it comes to evaluating the impact of natural compounds on histomonosis *in vivo* (Duffy *et al.*, 2004; Duffy *et al.*, 2005; Hafez & Hauck, 2006; Grabensteiner *et al.*, 2008; van der Heijden & Landman, 2008b).

Artemisia annua has been used as an herbal remedy in traditional Chinese medicine for treatment of fevers, including malaria (Klayman, 1985). Artemisinin is one of the main active components of this medicinal plant and has been shown to be effective against various protozoa, including *Plasmodium* spp. (Qinghaosu Antimalaria Coordinating Research Group, 1979) and *Eimeria* spp. (Allen *et al.*, 1997).

The aim of the present study was to examine the effect of a panel of extracts of *A. annua* leaves, as well as pure artemisinin, on *H. meleagridis* *in vitro* and *in vivo* using a standardized experimental infection model in turkeys (Hess *et al.*, 2006a).

Materials and methods

In vitro

Six different clonal cultures of *H. meleagridis* (Hm2, Hm3, Hm4, Hm6, Hm7 and Hm18) were used to evaluate the effect of artemisinin and *A. annua* materials. Clonal cultures were established through micromanipulation (Hess *et al.*, 2006b). Dry leaves from *A. annua* were finely ground before use. Artemisinin (purity > 99%) was obtained. Crude extracts from *A. annua* leaves were made using hexane, dichloromethane or methanol.

A test system previously described by Grabensteiner *et al.* (2007) was used. The concentration levels of the materials in the test cultures were: dry leaves, 5-40 mg/ml; artemisinin, 5-20 mg/ml; and hexane (Ext-HEX), dichloromethane (Ext-DCM) or methanol extracts (Ext-MeOH), 0.5-1.5 mg/ml. Protozoan growth and viability was evaluated after 24 and 48 hours of incubation at 40°C. The number of protozoa was ascertained using hemacytometer as described by Zenner *et al.* (2003).

In a similar setup, protozoa from *Histomonas meleagridis*/Turkey/Austria/2922-C6/04 (Hm6) were used to determine the inhibitory properties of artemisinin (IC₅₀) (10¹- 4×10⁴ µM). The IC₅₀ was enumerated by graphical extrapolation.

Bacteria present in the same monoeucaryotic *Histomonas* cultures were isolated using selective media. The antibacterial activity of the test materials were assessed using the disc diffusion method (Bauer *et al.*, 1966; CLSI, 2008). Test solutions (20 µl) in concentrations identical to those in the first experiment were loaded onto empty Sensi-discs.

The data analysis and statistical calculations were made using one way ANOVA followed by Tukey's multiple comparison test. *P*-values of ≤ 0.05 were considered as significant.

In vivo

Based on the results from the *in vitro* experiments an animal experiment was set up in order to investigate the effect of artemisinin and Ext-DCM on a virulent clonal culture of *H. meleagridis* (*Histomonas meleagridis*/Turkey/Austria/2922-C6/04) (Hess *et al.*, 2006a) in turkeys.

Sixty-five day-old turkey poults were randomly split into five groups with equal gender ratio (Table 1). From the first day of life the birds had unlimited access to feed (details mentioned below) and water, except a feed restriction for 5 hours directly after the infection. All procedures performed on the birds were approved by the institutional ethics committee and licensed by the Austrian government (licence number 68.205/0103-II/3b/2011).

Table 1: Overview of treatment groups

Group	Treatment	No. of birds
I	Ext-DCM 0.2% in drinking water (day 1-15); 0.1% (day 16-)	15
II	Artemisinin 100 ppm in feed	15
III	Artemisinin 2600 ppm in feed	15
IV Infection control	None	15
V Negative control	None	5

Administration of artemisinin was done via feed and Ext-DCM via drinking water. Supplementation started at first day of life and was continued throughout the experimental period. The dosage was determined due to the obtained *in vitro* results and followed the protocol of Grabensteiner *et al.* (2008). Artemisinin (purity > 99%) was added to the diets in concentrations of 100 ppm and 2600 ppm. Ext-DCM from dried *A. annua* leaves was dissolved in DMSO and administered to group I as follows: from day 1-15 received 0.2% pure extract in drinking water; from day 16 onwards the concentration was decreased to 0.1% due to reduced water intake. Fourteen days old, the birds (group I-IV) were infected intracloacally with 3×10^5 *Histomonas meleagridis*/Turkey/Austria/2922-C6/04 passage 22 (Hess *et al.*, 2006b). Birds of group III died or had to be euthanized before inoculation was undertaken.

Daily recordings of any adverse clinical signs, mortality, and feed and water consumption were obtained. Birds were weighed and had blood taken once a week. For monitoring the *H. meleagridis* excretion cloacal swabs were taken three times weekly according to the protocol described recently (Hess *et al.*, 2006a).

Turkeys that survived the challenge were killed at termination of the experiment at 5 weeks of age (Hess *et al.*, 2006a). Euthanasia due to severe histomonosis or killing of chickens at specific time points was performed by intravenous anaesthesia with thiopental followed by bleeding.

Pathological examination was performed on all birds. Lesions indicative for histomonosis in the caeca and the livers were noted with scores ranging from 0 for no lesions to 4 describing the most severe lesions, according to recently described protocols (Windisch & Hess, 2010; Zahoor *et al.*, 2011).

Results

In vitro

Addition of dry leaf plant powder resulted in complete growth inhibition at 20 and 40 mg/ml in Hm2, Hm3, Hm6, Hm7 and Hm18, whereas complete inhibition of cell proliferation was only seen at 40 mg/ml dry leaf powder in Hm4. The MLC for dry leaf powder was determined to 40 mg/ml after 24 h. For artemisinin, complete inhibition of protozoa proliferation was not observed in any of the six clones. However, significant reductions of histomonads were noticed after incubation for 48 h with artemisinin in all cultures. Growth of clonal cultures receiving 0.5 mg/ml Ext-HEX was significantly reduced in all six clones at 1.0 and 1.5 mg/ml Ext-HEX after 48 h. However, only 1.5 mg/ml resulted in complete inhibition in the six clonal cultures at 24 h of incubation. The MLC was determined to 1.5 mg/ml for Ext-HEX. When adding Ext-DCM to the protozoa cultures significant inhibitory effect against histomonads of all clones was observed at all three tested concentrations. Complete inhibition of the cultures after 24 h of incubation was observed at Ext-DCM levels of 1.0 mg/ml and 1.5 mg/ml (MLC= 1.0 mg/ml). In contrast to Ext-HEX and Ext-DCM, Ext-MeOH was not able to induce complete inhibition in any of the tested clonal cultures of *H. meleagridis*.

The IC₅₀ for artemisinin after 48 h was determined to 4.586×10^3 μ M, which equals 1.3 mg/ml in test solution.

No inhibitory effect of dry leaf powder, artemisinin, Ext-HEX, Ext-DCM or Ext-MeOH was observed in any of the 19 isolated bacterial strains from the six investigated *H. meleagridis* clones.

In vivo

At day 5-7 following feeding, 7 out of 15 birds from group III died unexpectedly. The remaining birds of this group displayed increasing depression and anorexia and were therefore euthanized. Post mortem section findings were suggestive of intoxication.

Furthermore, it was observed that the water containing 0.2% Ext-DCM (group I) had a very pronounced herbal odour. At 2 weeks of age the water intake of those turkeys decreased to 60-70% compared to turkeys of the remaining

groups. For welfare reasons based on the lower water consumption the concentration of Ext-DCM was hereafter reduced to 0.1% in group I.

Except from one bird in group II, *H. meleagridis* was found in cloacal swabs from all birds (group I, II and IV) at least once during the experiment. No histomonads could be recovered from any of the turkeys in group V.

All infected turkeys (groups I, II, IV) showed various clinical signs of histomonosis, from general depression and ruffled feathers to sulphurous coloured diarrhoea and sudden death. Turkeys that died or had to be euthanized due to histomonosis displayed severe disease specific lesions in the caeca and livers. Furthermore, necropsy of the 3 surviving turkeys revealed severe lesions in caeca and livers similar to pathological changes of turkeys that died from the disease (Table 2).

Table 2: Mortality and median hepatic and caecal lesion scores (LS) of turkeys suffering from histomonosis.

Group	Treatment	Mortality due to histomonosis (No. of birds)	Lesions specific to histomonosis (No. of birds)	Median LS	
				Liver	Caecum
I	0.2% (0.1%) Ext-DCM	13/15	13/13	4	4
II	100 ppm Artemisinin	13/15	15/15	4	3
IV	Infection control	13/14	14/14	4	3
V	Negative control	0/5	0/5	0	0

Discussion

The present *in vitro* experiment revealed significant dose dependant reductions in protozoal counts of all six tested clones of *H. meleagridis* for the tested concentrations of artemisinin and Ext-DCM. Although significant reduction rates were observed in all six clones, none of the artemisinin concentrations were able to induce a total inhibition of histomonad proliferation. Hence, no MLC could be determined. Based on the *in vitro* results, the IC₅₀ of artemisinin was determined to $4.586 \times 10^3 \mu\text{M}$, which is considerably higher than for malaria parasites, where IC₅₀ values are within the nanomolar range (Golenser *et al.*, 2006). Ext-DCM was the most effective leaf extract, displaying complete inhibition of protozoal multiplication at 1.0 mg/ml in all clonal *H. meleagridis* cultures.

At present histomonads need accompanying bacteria when cultured *in vitro*, however the role of the bacteria is not clear (McDougald, 2005). In the present study, the amount of artemisinin loaded onto the discs (100-300 $\mu\text{g}/\text{disc}$) had no antibacterial effect.

The results of the antiprotozoal screening and the antibacterial tests suggest that the observed inhibitory effect of dried *A. annua* leaves, artemisinin, Ext-HEX and Ext-DCM is due to a direct effect on histomonads. Ext-DCM and

artemisinin were found to have the strongest antihistomonal effect in the *in vitro* studies and were therefore selected for further *in vivo* testing.

Despite treatment with the Ext-DCM or artemisinin, the clinical outcome in turkeys was almost similar and of the same severity as noticed for the infected but untreated turkeys. Except for three birds, all infected turkeys died or had to be euthanized due to severe clinical signs.

An explanation for the discrepancy between *in vitro* and *in vivo* efficacy in the present investigation is unclear. It is possible that low bioavailability (Titulaer *et al.*, 1990) may contribute to insufficient concentrations of artemisinins in the birds. However, no information on the bioavailability and metabolism is yet available for poultry or other avian species.

The post mortem findings from the administrated 2600 ppm artemisinin group (III) may be indicative of intoxication and further investigations are on-going.

Conclusion

Despite considerable *in vitro* antihistomonal properties, neither artemisinin nor Ext-DCM of *A. annua* were able to prevent experimental histomonosis in turkeys at the given concentrations. Thus, the results of this study clearly demonstrate the importance of defined *in vivo* experimentations in order to assess and verify *in vitro* results.

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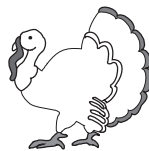
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